**MASTER'S THESIS**


# Long-Range PCR and PacBio sequencing, a novel method for detection of antibiotic resistance genes in class 1 integrons from waste water treatment plant.

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**Tiivistelmä – Referat – Abstract**

Antibiotics are used to prevent the microbial diseases in both animals and humans. Because of the overuse of antibiotics, the microorganism now gained the ability to resist the drugs through genetic changes. Integrons are widely known for their role in the dissemination of antibiotic resistance. The class1 integrons are mostly studied in Gram-negative bacteria of clinical strain as they are reported mostly in the human and animals. The integrons having antibiotic resistance genes are linked with mobile genetic elements which help them to disseminate by lateral gene transfer method. Previous research has proved that the class 1 integrons have sulfonamide and tetracycline resistance gene by using Long-Range PCR, Inverse PCR, and metagenomics. However, it is not clear what other possible combination of antibiotic resistance genes the class1 Integrons may carry. My thesis focuses on the class 1 integron from wastewater (both inflow and outflow water) by Long-range PCR, which can amplify fragments more than 15kb and PacBio RS long-read sequencing. It's a novel method of combining Long-range PCR and may illuminate what other possible resistance genes the class 1 integrons carry. The antibiotics resistance genes such as *CatB8, -aadA2, blaOxA-10, IMP-38* were amplified using our designed primers from *IntI1* to *QacEdelta1*, thus the designed primers and the optimization of Long-Range were successful. The combination of inverse PCR and Pac-Bio sequencing was successful to amplify the antibiotic resistance genes from Class 1 integrons. The Long-Range PCR saves time and gives DNA amplified products longer than 1500kb. The purified samples from long range PCR can be studied by direct sequencing using the Pac-Bio sequencer. Thus, the future implementations of the above combination of two techniques can be very useful to study the antibiotic resistance genes in the soil and polluted water. More in-depth information about antibiotic resistance genes in class 1 integrons will help to understand their dissemination.



| Avainsanat – Nyckelord – Keywords<br>PCR- Polymerase Chain Reaction, integron-integrase gene (IntI), recombination site (attI) and a promoter (PC), |
|---|
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## List of Abbreviations

1. **PCR-** Polymerase chain reaction
2. **TaKaRa LA PCR -** TaKaRa Long-Range Polymerase chain reaction.
3. ***intI1*-** The class 1 integron-integrase gene.
4. ***QacE*-** Quaternary ammonium compound-resistance protein
5. **NGS-** next-generation sequencing**.**
6. **EU**- European Union.
7. **ZMV-** zero-mode waveguides.
8. **SMRT**- Single Molecule, Real-Time.
9. **TAE**- a mixture of Tris base, acetic acid, and EDTA.
10. **IF**- inflow wastewater.
11. **OF**- outflow wastewater.
12. **IFP** – inflow wastewater pooled.
13. **OFP**- outflow wastewater pooled.
14. **NEG**- negative
15. **NCBI**- National Center for Biotechnology Information.
16. **BLAST-** Basic Local Alignment Search Tool.
17. **EMBL-** European Molecular Biology Laboratory.
18. **dNTPs-** deoxyribonucleotide triphosphate.
19. **MAFFT**-Multiple Alignment using Fast Fourier Transform.
20. **MUSCLE**-Multiple Sequence Comparison by Log-Expectation
21. **ARG-** antibiotic resistance genes
22. ***QacEDelta1*-** Quaternary ammonium compound-resistance protein delta 1
23. ***CatB3*-**chloramphenicol acetyltransferase

## 1. Introduction

Antibiotics were developed in the 1920s by the discovery of penicillin by Sir Alexander Flemings. In 1945 Fleming stated that "public will demand the drug and then will begin an era of abuses." [24,25]. Antibiotics are important for the advances in human medicine. Since then the resistance in bacteria is observed with maximum antibiotics that have been developed with later years. It is a major problem because the antimicrobial drugs will be ineffective while curing the diseases and cause in increase the global health problems such as causing prolonged illness and death[24].

Antibiotics are used to prevent the microbial diseases in both animals and humans[68]. The European Antibiotic Resistance Surveillance has noted that there is an increase in the resistance to the antimicrobial drugs like fluoroquinolones and carbapenems [33]

The antibiotic resistance happens when the bacteria can grow efficiently in the presence of the therapeutic antibiotics such as tetracycline (*tet*), trimethoprim resistance gene (*dfrA*), sulfonamide (*sul*). The bacteria can become resistant to fluoroquinones or carbapenems either by genetic mutation or by lateral gene transfer. The bacteria use horizontal gene transfer for transferring the genetic material between the neighboring bacteria. The horizontal gene transfer (figure-1) has three main processes: transformation, transduction, and conjugation.

**Transformation** is the direct uptake of shorter DNA fragments from the surrounding through cell membrane such as in *Haemophilus* spp. **Transduction** involves the bacteriophage which transfers the DNA by a bacteriophage as seen in *Escherichia coli*. **Conjugation** transfers the DNA fragments through a cell to cell contact. Thus, the bacteria can have multiple resistance genes due to these mechanisms and with time has gained the ability to resist more than one antibiotic.

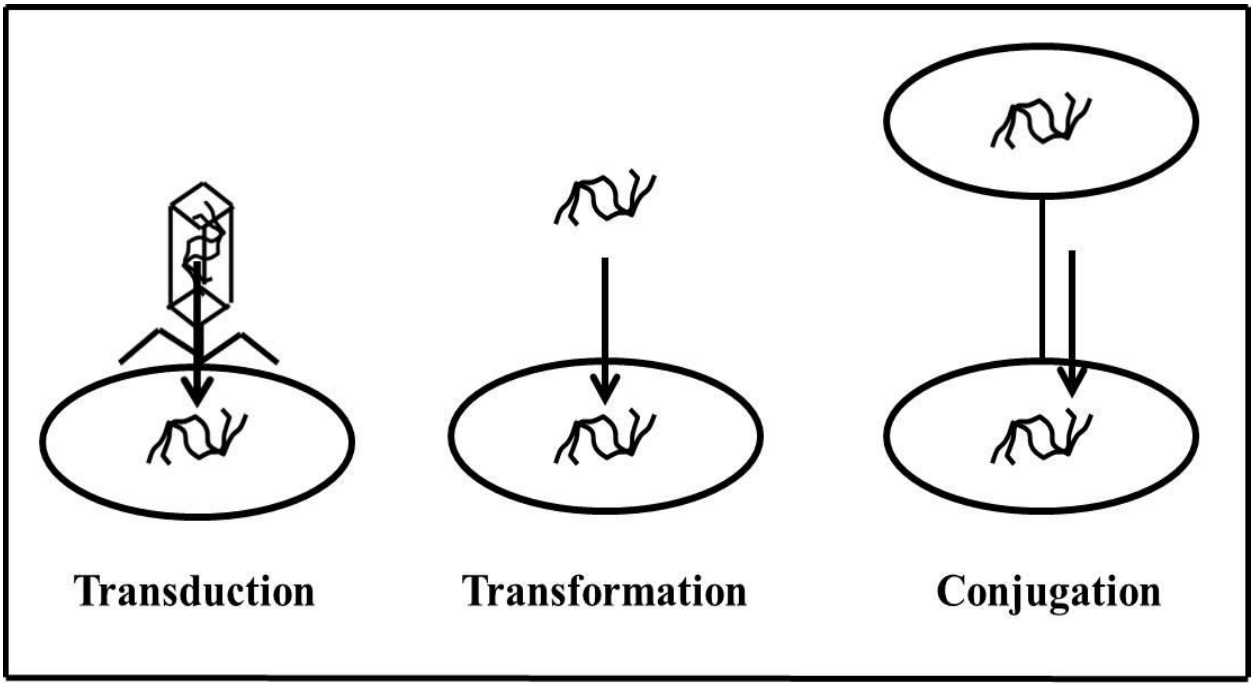


**Figure1: horizontal gene transfer.** Image credit: Wikipedia (2013)

By overexploitation of antimicrobial drugs, the bacteria can develop antimicrobial resistance by horizontal gene transfer mechanism. These antimicrobial drug resistance microbes are referred to as "superbugs"[36]. The resistance in the *Escherichia coli* can cause problems because of the overuse, the drugs now used in the treatment is ineffective in half of the patients due to resistant *Escherichia coli*. WHO (world health organization) has recently added gonorrhea as an emerging resistance to antibiotics [(2)] .

### 1.1 Antibiotic resistance around the world

The antibiotic resistance research studies are carried out worldwide. The most important countries are US, China, and Europe. Around 1243 antibacterial resistance research projects are carried out currently, with a total public investment in €1·3 billion across 19 countries of EU level[(29)]. The antibiotic resistance could be a major problem in the country's economy as in 2007 in Europe alone, around 25,000 people died from resistant bacterial sepsis which cost around €1·5 billion during that time [(29)]. A review in 2014 estimated that an additional 10 million lives a year will be lost by 2050 because of antimicrobial resistance in six key pathogens, out of that four are bacterial, resulting in a cost of US$100 trillion. The study on antibiotics have found that the usage of

antibiotics has risen by 36% from 10 years in 5 countries [50]. Research on the relation of integrons and drug resistance has been proven responsible for dissemination of antibiotic resistance among the clinical bacterial isolates. The article published by The Guardian states that 'Spain uses 100 times more antibiotics in intensive farming than Norway and Sweden since the routine mass medication is allowed in Europe. This has caused an increase in the use of antibiotics [26]

### 1.1 Antibiotic exploitations in Waste water treatment plants of Europe

The surveillance studies in EU conducted by a research group led by Dr. Goossens and colleagues on the antibiotics exploitation in Europe (at the University of Antwerp, Belgium) published that the antibiotic usage in Turkey are three times higher when compared to other European countries. They suggest that the antibiotics might be used illegally outside the hospital settings. The antibiotics are overused in farming and veterinary field in many countries such as Spain in Europe to promote the yield of the livestock [3]. The reason for this could be the human activities such as agricultural activities and urbanization have created a stress which accelerates the evolution and transfer of chromosomal resistance genes to mobile genetic elements causing the dissemination of the pathogenic strain [10]. The antibiotics waste which is discarded by the human activities such as farming is collected in the municipal wastewater or sewage systems and end up in the environment [27]. The antibiotics used for veterinary enter the environment through the manure [35]. Antibacterial drugs are also used for the fish farming [9], which can result in the high concentration of the of antibiotics in the fish farming water and sediments [28]. In Finland the farm soil has less antibiotic resistant gene due to annual freeze–thaw cycle and use of less manure. The adaptation of agricultural practice from Finland can lower the antibiotic resistance genes dissemination in soil [57]. Water bodies are the main vectors for the dissemination of the antibiotic resistance [8]. The prevalence of class 1 mobile integrons is high in polluted waters [8]. The wastewater treatment plants are the main hotspot in dissemination antibiotics resistance genes once discharged to rivers [69] as shown in Figure-2. The wastewater treatment plants process the polluted water for reuse. The bacteria and microbes are processed every day along with the wastewater in the treatmentplants making the wastewater water treatment plant as the main hotspot [70]. The processing of waste water through treatment plant includes UV-radiation and chlorination before releasing it to the rivers. Highly diverse bacterial biofilms are present in the wastewater treatment

plant [71] . Transduction and conjugation are the main mechanisms which helps in the exchange of resistance gene among the bacteria[72].

The high abundance of class 1 mobile integrons is associated with the sewage wastewater treatment where the river receives the outflow. In wastewater pollutant, antibiotic resistance genes to aminoglycosides, beta-lactams, resistance to trimethoprim [7] have been found.

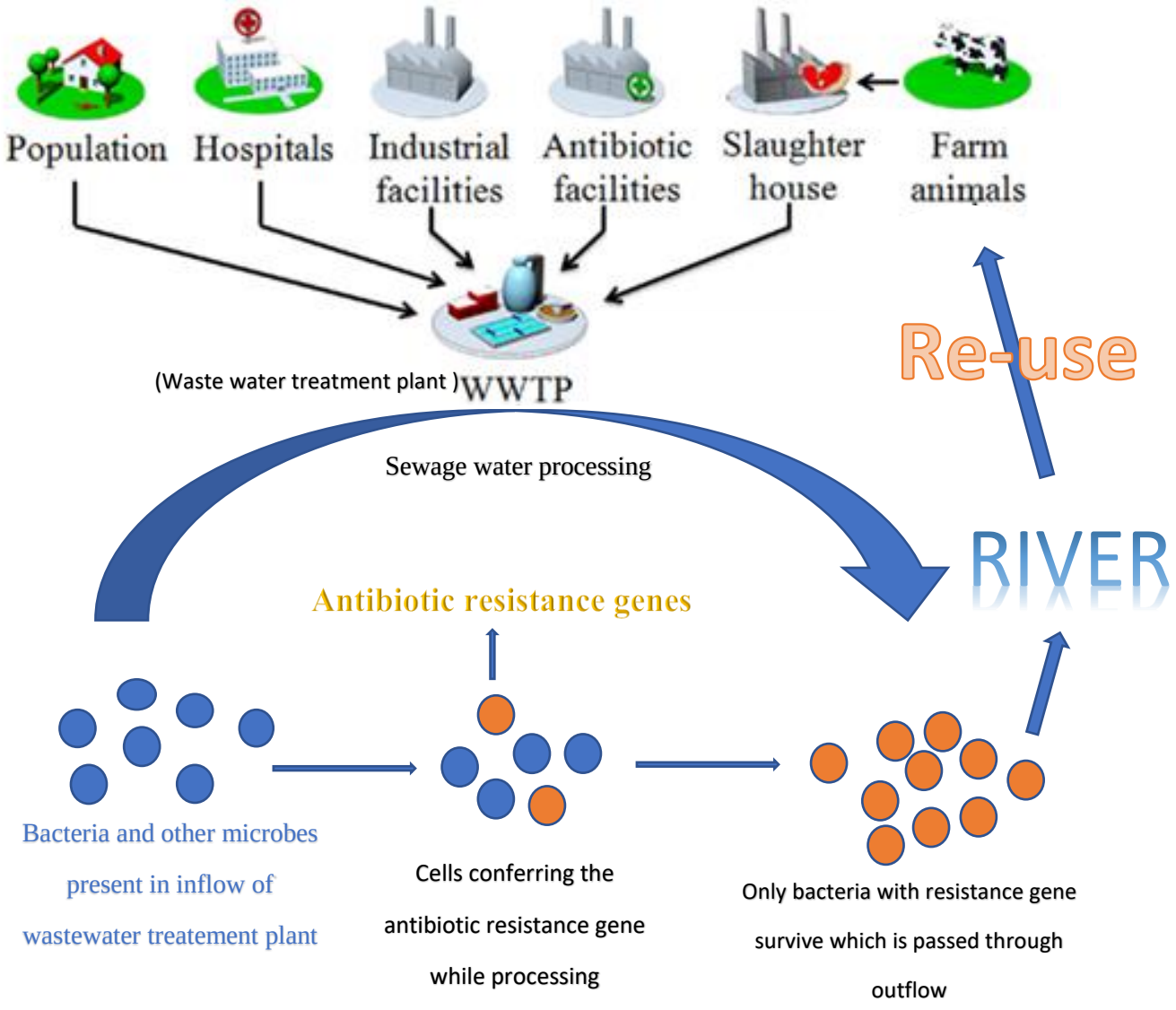


**Figure 2: wastewater treatment plant promoting the dissemination of the antibiotic resistance genes**

## 1.2 Integrons

The Integrons were 1st discovered in the 1980s as a result of studies that were carried on resistance plasmids and transposons due to overlapping antibiotic resistance genes [51,52]. Thus, the integrons helps in the dissemination of antibiotic resistance [51]. Based on the number of gene cassette of antibiotic resistance genes in the integrons, they are found in two categories: (1) mobile integrons and (2) chromosomal Integrons. The mobile Integrons have only few gene cassettes having antibiotic resistance genes cannot transport by themselves but are located on mobile genetic elements like transposons and plasmids. The mobile genetic elements include plasmids, bacteriophages, transposons. The chromosomal integrons have hundreds of gene cassette and are found in the chromosomes of bacterial genomes. They exhibit the same sequence of bacterial genomes obtained either from marine or terrestrial ecosystems[49]. They are known as Super-Integrons[53]. The integrons which are residing on the chromosomes are linked with mobile elements like transposons or conjugative plasmids [5].

Through the horizontal gene transfer, the bacteria can gain the mobile genetic elements having the multidrug resistance gene cassette. The bacteria can carry around 200 cassettes that can have proteins with unknown functions[61]. The integrons have three core features of integration (Figure 3): an integration-integrase gene (*intI*), a recombination site *(attI)* and a promoter (*PC*) [12]. These features allow the capture and express of exogenous genes as part of a gene cassette. Integrons having antibiotics resistance genes such as chloramphenicol, trimethoprim, β-lactams have been found as different combination in gene cassettes[62]. The genes present on the gene cassette are expressed from the promoter of integrons as the genes lack their own promoters[12].

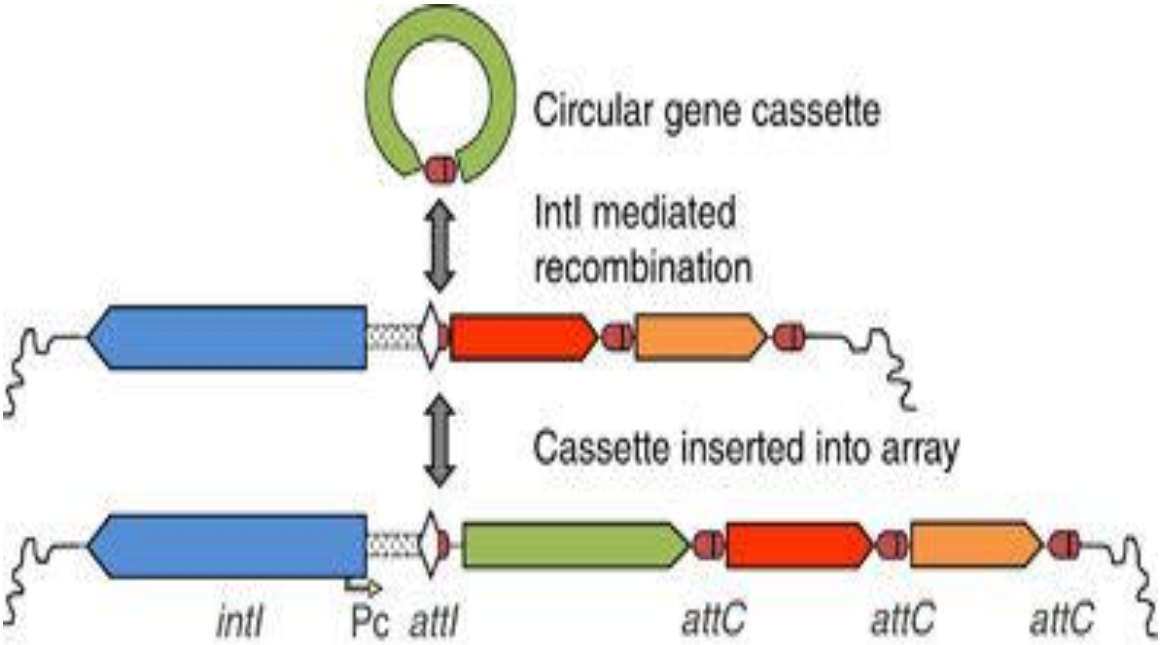


**Figure 3: Integron structure and function**. Image credits (Gillings et al.,2015)

The integrons are diverse in structural orientation. The movement of the integrons by horizontal gene transfer mechanism produce random genetic rearrangements and it is based on the principle of natural selection. This process of inserting new genes are due to cassette receptor site attI and deletion is done by the process of site-specific recombination between any of the 59 base elements [(42)]. According to their structural orientation the integrons are divided into 3 groups: The class 1 and 3 integrons are found from the freshwater and the soil environments (e.g. Proteobacteria). The class 2 integrons are present in the marine environments (e.g. gammaproteobacterial). The last is the reverse integrase integron which is limited to *Vibrio cholerae* from the environmental[42].

### 1.2.1 Class 1 integrons

Out of these groups, the Class 1 integrons has the presence of a 5′ conserved segment (CS). They are mainly reported in Gram-negative bacteria clinical strains mostly in the human and animals[62]. The 5' conserved region includes the DNA integrase gene, *intI1*[62]. The class 1 integron-integrase gene *(intI1*) from all such clinical isolates is highly conserved, exhibiting 99–100% nucleotide identity. The antibiotic resistance gene metallo-β-lactamase resistance to carbapenems has been reported in class 1 integrons from the clinical isolates of S*erratia marcescens* [(42)]. Integrons in Tn402 is known as an active transposon and most of the class 1 integrons are rearranged and seen as a defective derivative of Tn402 ancestral elements [(32,41)].

### 1.3 Environmental integrons

The mobile Integrons has been reported in natural ecosystems[58] . The class 1 integrons found from the environment are known as the environmental integrons. They are not usually linked with the mobile genetic elements, however, in recent findings of class 1 integrons the studies shows that the environment has a role in the spread of antibiotics resistance among the bacteria [6,54]. This can be due to overexploitation of the antibiotics by human activities such as aquaculture, agriculture, and industrial waste. They have put pressure on the environmental bacteria (Figure-4) to associate with the mobile genetic elements and transfer their resistances [4]. The mobile integrons have been found in the lake sediments[60]. Only a little research has been done on the mobile integrons from the soil sediments. A survey done on the forest soils has found that 11/24 strains of Enterobacteriaceae strains have class 1 mobile integrons[59].

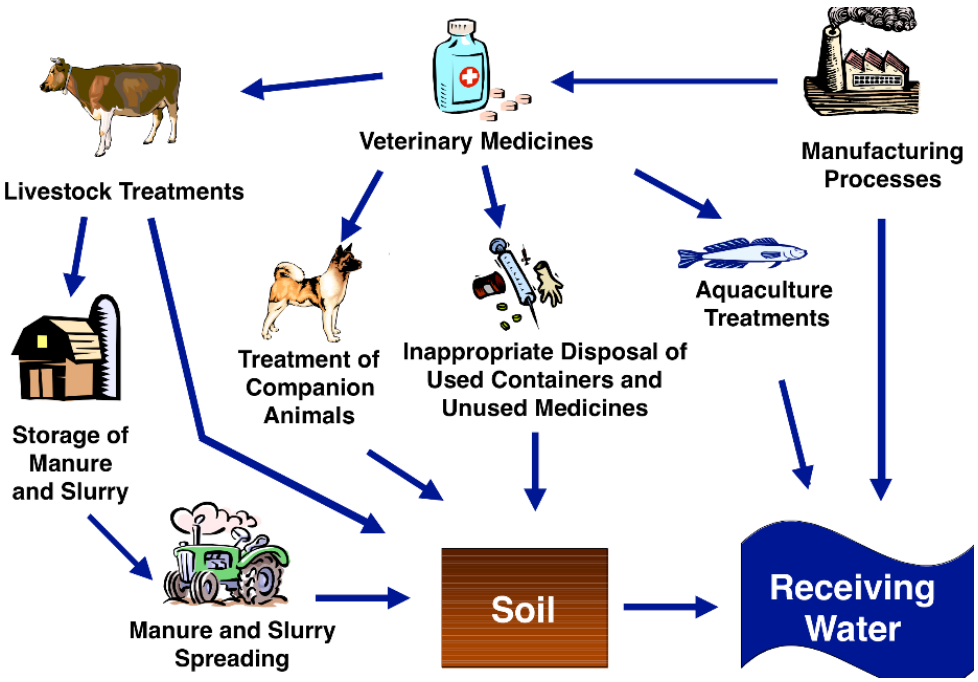


**Figure 4: Main route of Mobile Integrons dissemination from human sources to the environment** (pictures derived: https://toxics.usgs.gov/highlights/vet_meds.html)

### 1.4. Long-Range PCR

The efficient amplification of the long regions of DNA always been a problem with standard PCR. However, in the Long-Range PCR, the polymerase is modified such that it could amply around 30 kb DNA fragments [46]. The DNA amplification by Long-Range PCR combined with next-generation sequencing is considered as an effective tool for analyzing the long DNA sequences [55,56].

### 1.5 Nested PCR

PCR with newly designed primers will give impure product and low yield of the desired amplicons [11]. Nested PCR is a better solution that can enhance the pure products and yield of the desired amplicons.

This method requires two pairs of PCR primers the first set (outer primers) binds to the target sequence of interest and after conventional PCR, we get a product that has both the target sequence and non-specific sequences because the sample materials might contain numerous bases to which our primer can bind. To have a high selectivity of the product, a second set of barcoded primers (nested primers) are used (Figure 5) for sequencing. These primers will bind to the precise region of the target sequence [34]. The product from the 1st set primers will be used as a template for the 2nd set of primers. Nested PCR can increase the yield of the desired target with a limited amount of DNA samples [23].

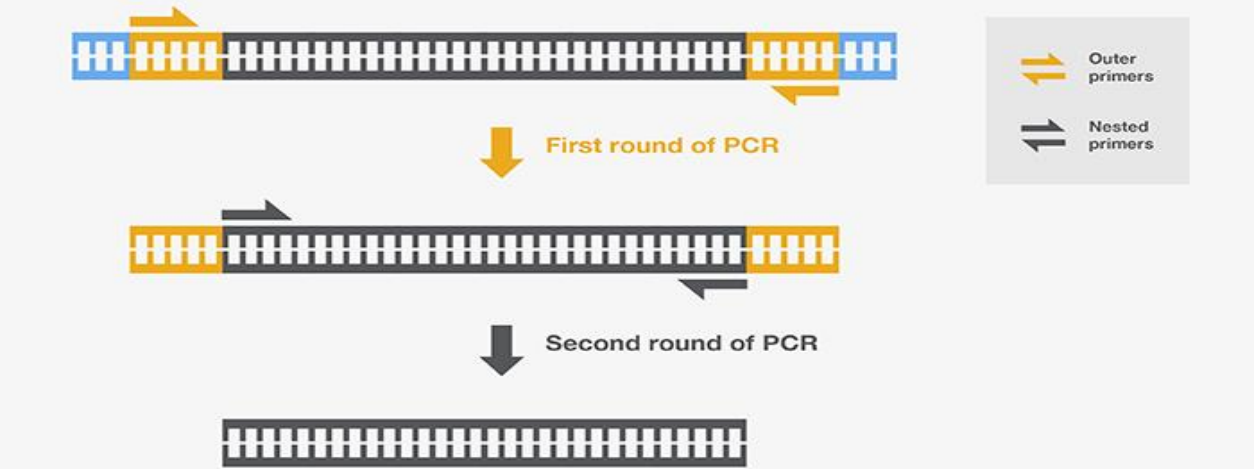


**Figure 5: Nested PCR credits:** Thermofisher scientific

### 1.6 Next generation sequencing

The next generation sequencing [16, 18] allows sequencing thousands to million molecules of DNA molecules. It is an important tool for the personalized medicine, genetic diseases. Because of the high throughput option it has a capability for multiple sequencing.

In next-generation sequencing, the machines have been developed using different technical details, though they have some common features like sample preparation as all NGS requires library obtained by amplification or adapter sequences ligation. The library fragments are then amplified on a solid surface having DNA linkers and hybridize the library adapters and produce clusters of DNA and each cluster will be considered as an individual sequence reaction. Then comes the DATA output, which will provide the raw data which is a DNA sequence generated at each cluster.

#### 1.6.1 The SMRT sequencing – Pacific Biosciences

The PacBio has an advantage of real-time acquisition, highly sensitive, high-speed sensors and cameras. The DNA polymerase can read around 1000bp/Sec makes PACBIO SMRT technology makes it a better choice for whole genome sequencing [37]. In Pac-Bio sequencing, the single molecules in real time (SMRT) cells have around 10,000 of zero mode waveguides which has the smallest detection volume with less background noise (Figure 6). During the process, the ZMVs are illuminated from their below surfaces and the light wavelength is too large to pass through the wave guide[13,14,17]. Thus ZMVs create the most powerful microscope of having a detection volume of 20 zeptoliters because the attenuated lights penetrate from the below of 20-30nm of each ZMV[15]. In this, the DNA template and polymerase are immobilized at the ZMV in the bottom (parallel to 1000s of ZMV). The DNA template and polymerase are introduced to the phospholinked nucleotides labeled with a different color fluorophore. When a base is added, the light is emitted. After the phosphate is attached, the phosphate chain is then cleaved releasing the attached fluorophore.

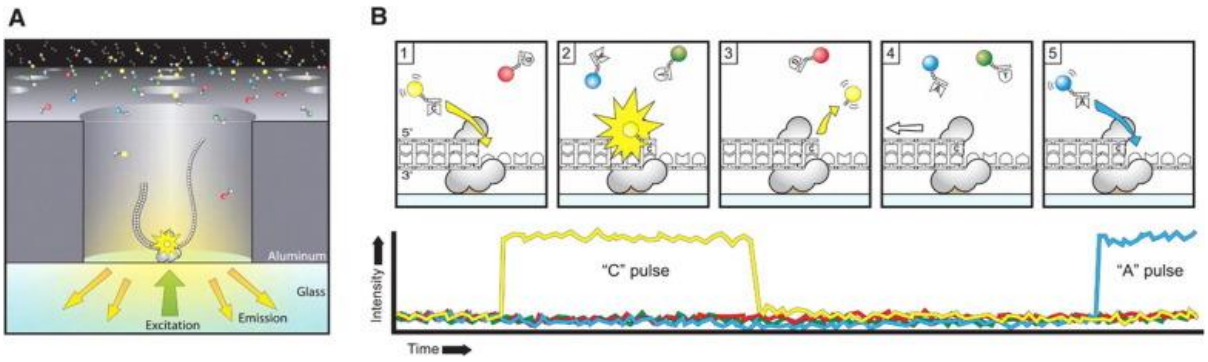


**Figure 6: The SMRT sequencing – Pacific biosciences** (image credits: AnthonyRhoads[1], Kin FaiAu.,2015)

The genome sequencing is popular with PacBio. It requires a high molecular weight DNA, which is then fragmented to a given insert size. The library prep (figure-7) includes the addition of specific PacBio adapters ligated at the ends of the insert (SMRT bell) [38]. The adapter act as a priming site where the polymerase binds and starts to sequence the insert. There are two types of inserts as per the following:

a) Short insert: which is around 100-2,000 bases long known as Circular Consensus Sequencing (CCS). The insert is read multiple times. (Mostly used for 16s sequencing)
b) Long insert: around 2,000-10,000 bases long known as Continuous Long Reads (CLR).

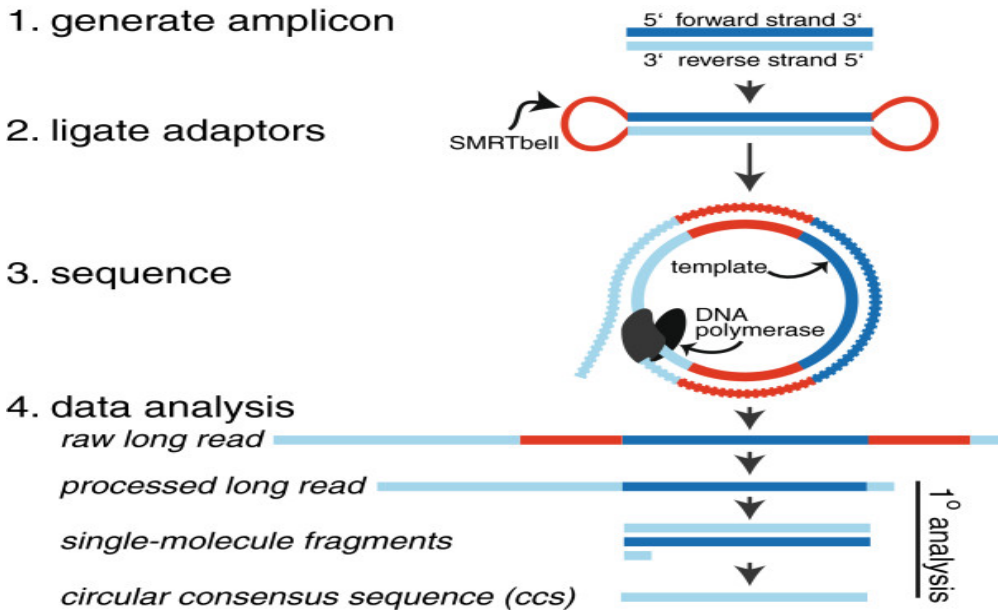


**Figure 7: Illustration of the PacBio library preparation**. Image credits - Fichot and Norman Microbiome 2013.

## 2. Objectives

This thesis focuses on the class 1 integron, from wastewater treatment plant (both inflow and outflow water) as sample. Sample is amplified by Long-range PCR, which can amplify fragments more than 15kb[46]. Amplified samples are used for PacBio RS long-read sequencing. Its a novel combination method and may illuminate what other possible resistance genes the class 1 integrons carry in the waste water treatment plant sample.

## 3. Materials

1. **DNA extraction from wastewater**
    a. DNeasy® powerwater® kit
    b. Wastewater sample (inflow and outflow)
    c. Filter funnel
    d. Filter membrane
2. **Long-range PCR**
    a. TaKaRa LA PCR™ Kit
    b. PCR tubes
    c. Primers
3. **Agarose Gel-electrophoresis**
    a. 0.5% agaose in 120ml 1X TAE buffer (0.6gms in 120 ml 1X Tris base, acetic acid and EDTA(TAE) buffer)
    b. Ethidium bromide
4. **Sequencing**
    a. PacBio RS long-read sequencing (The Pacific Biosciences sequencing were done by Institute of Biotechnology, University of Helsinki, Viikki campus, Helsinki, Finland.)

## 4. Methods

### 4.1 Primer designing for Long-range PCR

For designing the primers specific for the integron integrase genes *intI1*, the gene sequences of *IntI1* gene and *QacEdelta1* derived from Klebsiella pneumonia integron class 1 DNA, strain: MBL-09 were blasted using NCBI blast. The multiple sequence alignments were done using clustalW which is a tool of European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI) and made a consensus sequence. With this consensus sequence, the primer blast was done against *IntI1* and *QacEdelta1*. Further to check if the designed primers bind to the correct sequence of *IntI1* and *QacEdelta1* gene, blast search of just the primer sequences were done using the PRIMER-BLAST. The primers were blasted using CSC's supercomputer Taito to check if my primers bind to the sequences available on that server. Figure 8 illustrates the primers designing flow chart of *intI1* gene sequences and *QacEdelta1* from *Klebsiella pneumonia*.

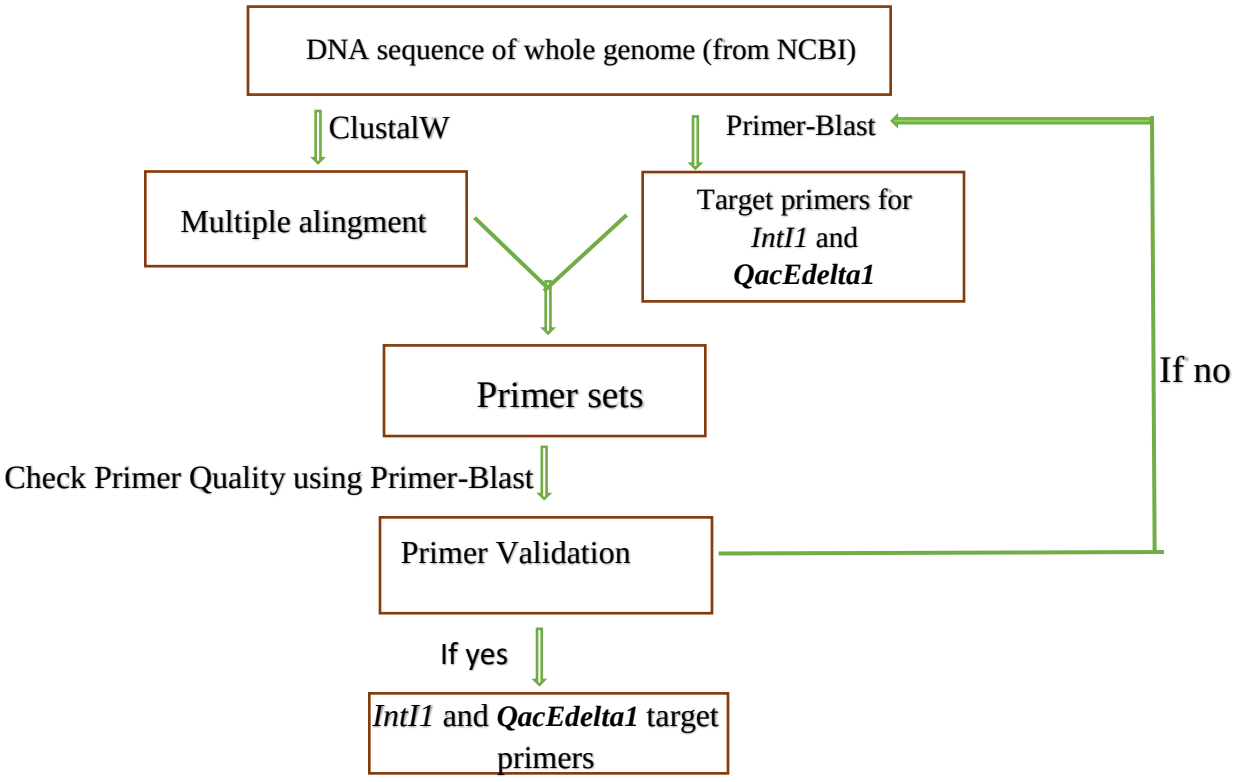


**Figure 8: Flow chart of Primers designing**. Primers for *IntI1* and *QacEdelta1* gene sequences using PRIMER-BLAST (NCBI).

Table 1: the sequence of primers used in Long-Range PCR.

| name | Sequence | Tm | Reference |
|---|---|---|---|
| Inti1p1 fv* | GCA GTT GCA AAC CCT CAC TG | **60** | This study |
| Inti1p1 RV* | GAG GCA TTT CTG TCC TGG CT | **60** | This study |
| int1 p2 FV* | TCG TGA TGC CTG CTT GTT CT | **58** | This study |
| int1 p2 RV* | AC TGC GGG TCA AGG ATC TG | **60** | This study |
| int1 p3 FV* | AGA TCC TTG ACC CGC AGT TG | **60** | This study |
| int1 p3 RV* | GTCC TCG GTT TTC TGG AAG GC | **60** | This study |
| int1p5 RV* | GTT TGT TCG CCC AGC TTC TG | **60** | This study |
| int1 p5 FV* | CAG ATC CTT GAC CCG CAG TT | **60** | This study |
| int1p6 FV* | CAT CGT CGT AGA GAC GTC | **63** | This study |
| int1 p6 RV* | AGA ACA AGC AGG CAT CAC GA | **58** | This study |
| int1 p7 FV* | AGC CAG GAC AGA AAT GCC TC | **60** | This study |
| int1 p7 RV* | TTC ATC CGT TTC CAC GGT GT | **58** | This study |
| int1 FV array1+ | GCC TTG ATG TTA CCC GAG AG | **60** | mmx |
| int1 RV array1+ | GAT CGG TCG AAT GCG TGT | **56** | mmx |
| int1 FV array 2+ | GAC GGC TAC CCT CTG TTA TCT C | **64** | mmx |
| int1 RV array2+ | GCC ACC ACT TGT TTG AGG A | **57** | mmx |
| int1 FV array 3+ | TGC TTT TCC CAC CCT TAC C | **57** | mmx |
| int1 RVarray 3+ | GAC GGC TAC CCT CTG TTA TCT C | **64** | mmx |

| | | | |
|---|---|---|---|
| intI1F165PA FV‡ | CGA ACG AGT GGC GGA GGG TG | **67** | 57 |
| intI1R476PA RV‡ | TAC CCG AGA GCT TGG CAC CCA | **65** | 57 |
| INT3-02 FV*1 | GCC ACC ACT TGT TTG AGG A | **57** | This study |
| INT3-02 RV *1 | GGA TGT CTG TGC CTG CTT G | **59** | This study |
| Qac E FV pa ‡ | GCC CTA CAC AAA TTG GGA GA | **58** | 45 |
| QacE RV pa ‡ | CTG CGG TAC CAC TGC CAC AA | **63** | 45 |
| Qace FV* | TCA TAA TTG CTG CCT TTT TGC TCG | **62** | This study |
| qace RV1* | GGC TAG GGG TAG CAC CTT | **58** | This study |
| qace FV2 kta * | CCC CTT CCG CCG TTG T | **56** | This study |
| qace FV2 kta * | CGA CCA GAC TGC ATA AGC AAC A | **62** | This study |

┼-primers from MMX group -Metals, microbes and xenobiotics - interactions and biotechnology group , University of Helsinki.

*- primers from NCBI primer-blast

‡- primers referred from scientific papers

### 4.2 DNA extraction and quantification from wastewater

The DNA extraction is done from the wastewater provided by Viikinmäki wastewater treatment plant located in Helsinki. The DNA was extracted from 1l of both inflow and outflow wastewater samples and kept the DNA samples in -20 freezers for further analysis. The DNA extraction of wastewater is done by DNaesay power water kit procedure (QIAGEN)[20].

According to the manufacturers instruction, the wastewater samples were filtered using a filter funnel attached to a vacuum source. Now use two sets of sterile forceps and pick up the white filter membrane and roll into a 5 ml Power Water DNA Bead Tube. Add 1 ml of PW1 Solution to the

Power Water DNA Bead Tube and vortex it at the maximum speed for 5 min. Centrifuge the tubes ≤4000x g for 1 min at room temperature. Transfer the supernatant to a 2 ml Collection Tube. Centrifuge the collection tube at 13,000 x g for 1 min at room temperature then supernatant to a clean 2 ml Collection Tube. Add 200 µl of IRS Solution and vortex it. Centrifuge the tubes with IRS solution at 13,000 x g for 1 min and transfer the supernatant to a clean 2 ml Collection Tube. Add 650 µl of PW3 Solution and vortex it. Transfer 650 µl of supernatant onto an MB Spin Column and Centrifuge at 13,000 x g for 1 min. Place the MB Spin Column Filter into a clean 2 ml Collection Tube and add 650 µl of Solution PW4. The sample was then centrifuge at 13,000 x g for 1 min. Discard the flow-through and add 650 µl of ethanol to centrifuge two times at 13,000 x g for 1 min. Place the MB Spin Column into a clean 2 ml Collection Tube. Add 100 µl of Solution EB to the center of the white filter membrane and centrifuge the MB spin column with EB solution at 13,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for is ready for PCR. The results of the amplification reaction were visualised by agarose gel electrophoresis.

### 4.2.1 Determination of DNA concentration

The DNA quantification was done by two methods, 1st is the NanoDrop and 2nd is the Qubit with a detection limit of 2 and 0.1 ng $\mu l^{-1}$ respectively. The outflow (OF) has four DNA samples extracted from wastewater outflow and inflow (IF) has four DNA samples extracted from Inflow wastewater (The filtering was done four times).

## 4.3 Long-Range PCR of wastewater to validate the primer quality

The Long-Range PCR of the wastewater sample from treatment plant with the primers was done by using TaKaRa LA PCR™ Kit Ver. 2.1. The protocol for PCR was provided with the kit [21]. After the DNA extraction, the Long-Range PCR was done using the PRIMESTAR protocol with the freshly the extracted DNA from wastewater samples (kit used TAKARA clontech) following conditions:

Table-3 Long-Range PCR condition

| PCR Conditions For ≦10 kb products | | |
|---|---|---|
| 98℃ | 10 sec | 30 cycles [3-step PCR] |
| 60℃*1 | 15 sec | |
| 68℃*2 | 10 sec/kb | |

* 1: if the Tm value is more than 55℃, the annealing temperature is 60℃.

* 2: For 3-step PCR, set the extension temperature to 68℃.

The samples were kept in +4℃ freezer for further use. The PCR reaction mixture components with volume are provided in the Table-2.

This procedure was repeated until all my primer quality was analyzed. After the long-range PCR. The PCR product was analyzed by the agarose gel electrophoresis (0.5% agarose in TAE buffer, 100v for 45 minutes)

Table-2: composition of PCR reaction mixture for Long-Range PCR

| **Components** | **Volume (**µl) | **Final concentration** |
|---|---|---|
| Primer buffer | 10 | 1X |
| dNTP Mixture (2.5mM each) | 4 | 200µM each |
| Primer 1 | 1.5 | 0.2-0.3 µM* |
| Primer 2 | 1.5 | 0.2-0.3 µM* |
| Template | 1 | |
| Prime star GXL DNA pol | 2 | 2.5U/50µl |
| Water | 30 | |
| Total volume | 50 | |

*When amplifying products ≧10 kb in length, use primers at a final concentration of 0.2 µM each

### 4.4 Optimization of the Long-Range PCR with wastewater sample

Different protocols for Long-Range PCR was followed to get high yield DNA from wastewater samples. The 1st change was to reduce the amount of template. The template volume for the PCR reaction mixture was changed from 1 µl to 0.5ul (27.4375 nanograms). The 2nd change was to use TaKaRa long range kit because the TaKaRa long-range PCR kit was used to amplify the DNA fragments more than 10kb products. Other changes includein the electrophoresis condition. The running voltage was reduced the from 100 volts to 90 volts for 1 hour.

The optimization has improved the DNA yield with Long-Range PCR and gave better amplification range from 3000-5000kb bands from the wastewater DNA samples. The PCR cycling conditions were as follows:

| PCR Conditions | | |
|---|---|---|
| 98°C | 10 sec | |
| 94°C | 3 mins | |
| 94°C | 15 secs | |
| 62°C*1 | 30 sec | 30 cycles [3-step PCR] |
| 68°C | 10 sec/kb | |
| 72°C | 10 mins – extension | |
| 4°C | ▸ | |

∗ 1: if the Tm value is more than 55°C, the annealing temperature is 62°C.

### 4.5 Nested PCR

Two different sets of primer are used with sample DNA samples. The DNA is extracted from the agarose gel that had amplicons after 1st Long-Range PCR. This PCR product from the gel is used for the second PCR with barcoded primers. The DNA is extracted from the gel using GeneJET™ Gel Extraction Micro Kit (producent: Thermo Scientific). The procedure was followed by the kit manual.

### 4.5 Sequence analysis

The best samples of PCR amplicons having highest yield (using primers specific for *intI1* gene as forward and *QacEdelta1* as reverse) was sent for the sequencing. The purified samples along with the best primers giving a band length of 1500 kb – 5000 kb long in electrophoresis with the barcoded primers were sequenced in Institute of Biotechnology, University of Helsinki, Viikki campus, Helsinki, Finland. The sequence analysis was done using the Galaxy and Geneious bioinformatics tools. I followed the de-novo sequencing method, where the query sequence is mapped against the reference genome_Escherichia coli strain CRE1540 chromosome, complete genome.

The alignment and clustering were done with same reference genome (Escherichia coli strain CRE1540 chromosome, complete genome. ) which is used for designing the primers against *intI1* and *QacEdelta1*. I got in total 2014 CCS. After quality control and quality filtering using Galaxy web-based platform, I made 3 consensus sequences of *QacEdelta1* and *IntI1* respectively using the MAFFT tool. The clustering was done using USEARCH with 90% clearance. The consensus sequence was blasted using megablasthit. The consensus sequences length was more than 1200bp long. The workflow of sequencing was followed from previous work of Pärnänen et al. on class 1 integrons using Inverse PCR[(1)].

The consensus sequence was made by running multiple sequence alignment MAFFT (Multiple Alignment uses Fast Fourier Transform) with the following parameters as in table 5. It is faster as compared to the Geneious alignment and simpler to MUSCLE (Multiple Sequence Comparison by Log-Expectation) as it requires Linux. The consensus sequence is saved in text format for further use.

Table 3: Multiple alignments with MAFFT

| **Parameters** | |
|---|---|
| Direction of nucleotide | Same as input |
| Output order | aligned |
| Try to align the gappy regions | yes |
| Scoring matrix for amino acid | BLOSUM62 |

| | |
|---|---|
| Scoring matrix for nucleotide sequence | 200PAM/ k=2 and with 1PAM/k=2 (closely related DNA sequences. |
| Gap opening penalty | 1.53 |
| Offset value | 0.0 |
| Scores of N in nucleotide data | nZero(N has no effect) on the alignment score |

### 4.5.1 Clustering and Blast search of the sequence from Pac-Bio sequencing

Clustering of the reads was done using USEARCH (UCLUST) with the reference genome sequence as shown in the table-12. Clusters had a clearance of 90% and had 54 clusters. The reference genome used in clustering for *IntI1* and for *QacE delta 1* was Escherichia coli strain CRE1540 chromosome, complete genome.

# 5. Results

## 5.1 Long-range PCR of wastewater treatment to amplify DNA having class1 integrons

The DNA sample from waste water treatment plant quantification was done the NanoDrop. One microliter of each sample was loaded in NanoDrop directly after extraction. The concentration of DNA is provided in Table-4.

Table-4: DNA quantification using Nanodrop.

| **Outflow (NANO DROP) ng/µl** | **Inflow (NANODROP) ng/µl** |
|---|---|
| 60.3 | 56.7 |
| 60.7 | 43.6 |
| 42.7 | 54.8 |
| 55.8 | 75.8 |

### 5.1.1 Long-Range PCR of wastewater treatment plants samples to validate the primers.

To check the specificity of primers designed for long-range PCR of Class 1 integrons was done with the DNA samples obtained from the wastewater treatment samples (inflow and outflow from Viikinmäki wastewater treatment plant located in Helsinki). The primers were used to amplify *IntI1* and *QacE* genes. The primer as shown in the Table-1, successfully amplified the DNA fragments of length from 1500kb-5000kb as shown in Figure-9 and Figure-10.

Three replicates of inflow and outflow are used respectively for the long-range PCR in Figure-9. The DNA samples gave a high yield band ranging from 5000kb-1500kb length. The DNA samples of outflow and inflow wastewater treatment plants were pooled together respectively to decrease the number of samples.

Total 30 primers from Table-1 with different combinations were checked for the DNA samples from wastewater treatment plants by Long-range PCR. The best 3 sets of primers are chosen for the further study, while rest of the primers were discarded.

The 1st primer set includes IntI1-p6fv and Qace rv2 kta which can successfully amplify the DNA from 1500kb-5000kb. The 2nd set of primer which includes Int1 fv array2 and QacE rv pa can amplify the DNA from 500kb -3000 kb and the final set of primer includes int1 fv array1and QacE rv1 amplified the DNA from 500 kb -5000 kb.

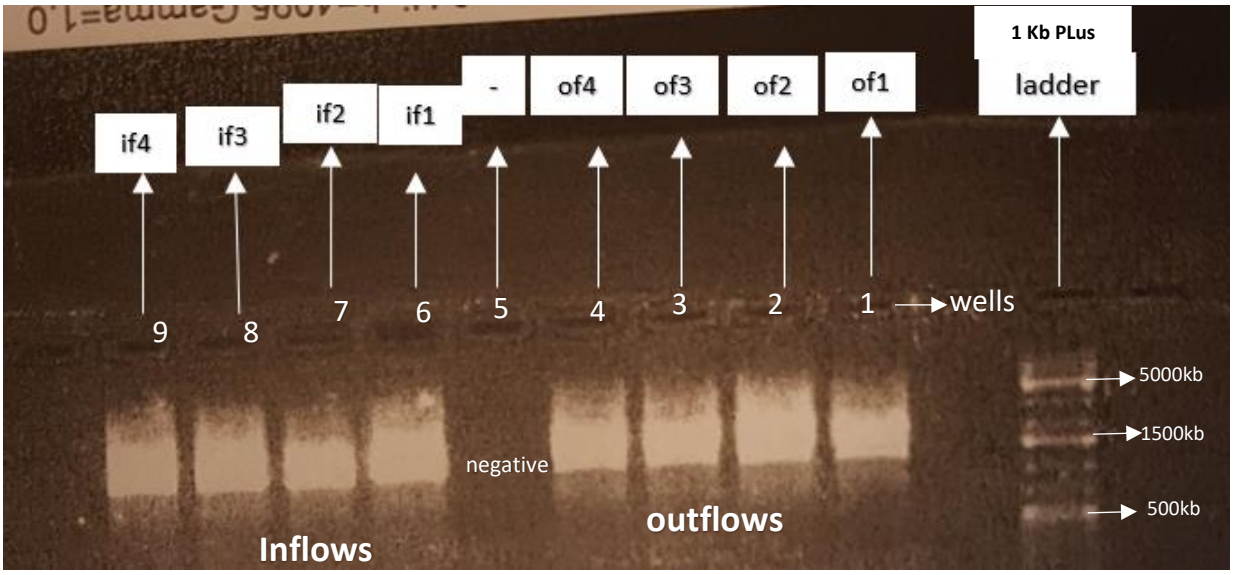


**Figure 9: Electrophoresis of Long-range PCR products**. Lane 1-4 is the outflow(of), 6-9 is the inflow (if). Primers used are IntI1-p6fv and Qace rv2 kta.

### 5.1.2 Optimized Long-Range PCR to amplify class 1 integrons

Optimization of the Long-Range PCR was done to get high yield DNA products from wastewater samples. Changes in the protocol include reducing the amount of template, increase the annealing temperature, reduce the number of PCR cycles, electrophoresis voltage, and buffer and performing PCR with the Long-Range kit. The primer used for H1 is Int1array2 and QacErvpa which amplified the DNA from 500kb -3000 kb and the primer used for H2 are Int*I1* primer array1 and Qacerv1 amplified the DNA from 500 kb -5000 kb.

The optimized PCR cycling conditions were as follows:

| PCR Conditions | | |
|---|---|---|
| 94℃ | 3 mins | |
| 94℃ | 15 secs | |
| 62℃*1 | 30 sec | 30 cycles |
| 68℃ | 10 sec/kb | |
| 72℃ | 10 mins – extension | |
| 4℃ | ▸ | |

∗ 1: if the Tm value is more than 55℃, the annealing temperature is 62℃.

The protocol was successful in amplifying the DNA of interest with a band with ranging from 400kb to 3000kb in both h1 box and h2 box. The Figure-10 represents the primer Int*I1* primer array1 and Qacerv1 which successfully amplified the DNA from 500 kb -5000 kb.

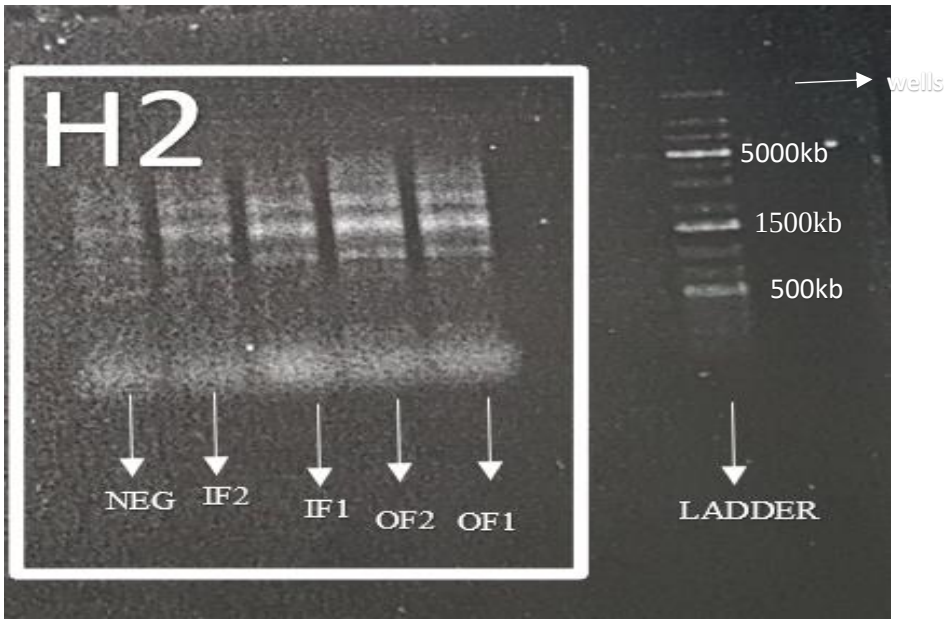


**Figure 10: Electrophoresis of Optimized Long-Range PCR for Wastewater treatment DNA**. (OF) is the outflow and (IF) is the inflow. (-) is negative without any templates. Primers used *IntI1* array1 and Qacerv1.1kb plus ladder concentration- 0.5µl

### 5.2 Nested PCR

The samples were purified from Figure-10 gel and are used for Nested PCR with barcodes to amplify the longer bands from the Long-Range PCR. The gel was made with 1.5% agarose and was ran on 100V for 45 minutes. The set 1 box of Figure -11 includes primers int1 fv array1 and QacE rv kta with barcodes. The set 1 primer amplified 1500kb of purified DNA sample from Figure-10 while the set 2 box of Figure-11 includes primers IntI1fv array2 and QacE pa which failed to amplify any bands

Table 5: primers used in Nested PCR

| Primers | set |
|---|---|
| int fv array1<br>QacE rv kata | 1 |
| IntI1fv array2<br>QacE paper | 2 |

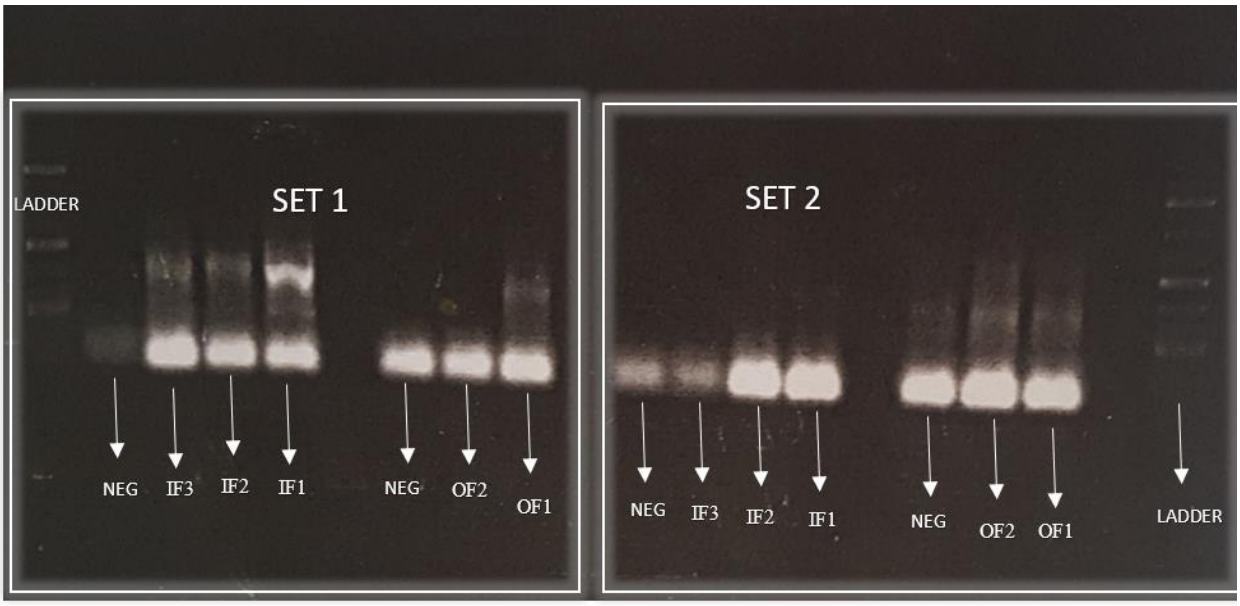


**Figure 11: Nested PCR**. set 1 box with set1 primers and set 2 boxes has Set2 primers. OF is the outflow IF1 is the inflow. NEG is negative. The ladder is 1KB plus.

### 5.3 Pac-Bio Sequencing

Total 2104 reads of Pac-bio sequences obtained from PacBio SMRT cell sequencing. The reads are divided into two parts. The 3 read in which the polymerase used in Pac bio will read from 3'-5' and the 5 read which the polymerase will read from 5'-3' direction. The quality control and quality filtering were done using the Galaxy project. The galaxy project is a web based scientific analysis of accessible data, which is completely reproducible. Out of 1089 sequences of 3 reads 795 passed the quality test and out of 1015 sequences of 5 reads 795 passed the quality test (mean PHRED score 41, length distribution length distribution 99-2999, mean length is 1700). The sequence files in the FASTAQ file or FASTA format were converted using fastq2fasta pro. Three consensus sequences of *IntI1*gene and *QacE delta 1* using Geneious[73] bioinformatics tool.

#### 5.3.1 characterization of class 1 integron genes from *IntI1* to *QacEdelta1*

The antibiotic resistance genes found in the class 1 Integrons from the DNA samples of wastewater treatment plant includes *dfrA12* gene (trimethoprim-resistant dihydrofolate reductase DfrA12 CDS), *aadA2* CDS gene (resistance to streptomycin/spectinomycin), *blaVIM-2* CDS gene (metallo-beta-lactamase), trimethoprim, metallo-beta-lactamase, streptomycin were present between *IntI1* gene to *QacEdelta1* gene. The presence of attI and attC proves the accumlulation of the new antibiotic resistance gene cassette in the variable region of integron. attC site specific recombination *gcuF* gene encode hypothetical protein[74] was found in Class 1 integron of our wastwater treatment plant sample as shown in Figure-12.

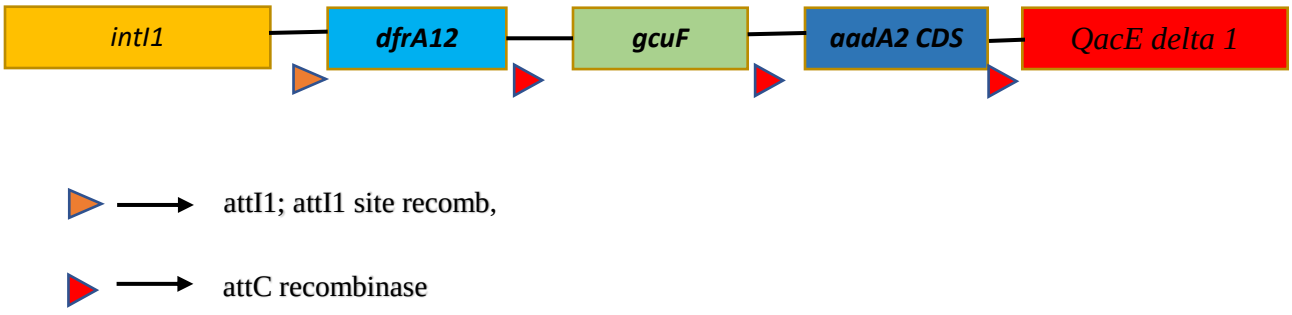


Figure 12: Illustration of the gene *dfrA12 – gcuF – aadA2* in class 1 integron found between *IntI1* to *QacEdelta1* by a site-specific recombinase.

The antibiotic resistance genes such as trimethoprim, metallo-beta-lactamase, streptomycin were observed between *IntI1* to *QacEdelta1* from the sequence analysis of DNA samples from wastewater treatment. The different gene cassette in class 1 integrons with their antibiotic resistance from *IntI1* to *QacEdelta1* obtained from wastewater treatment plants are shown in the Figure 13.

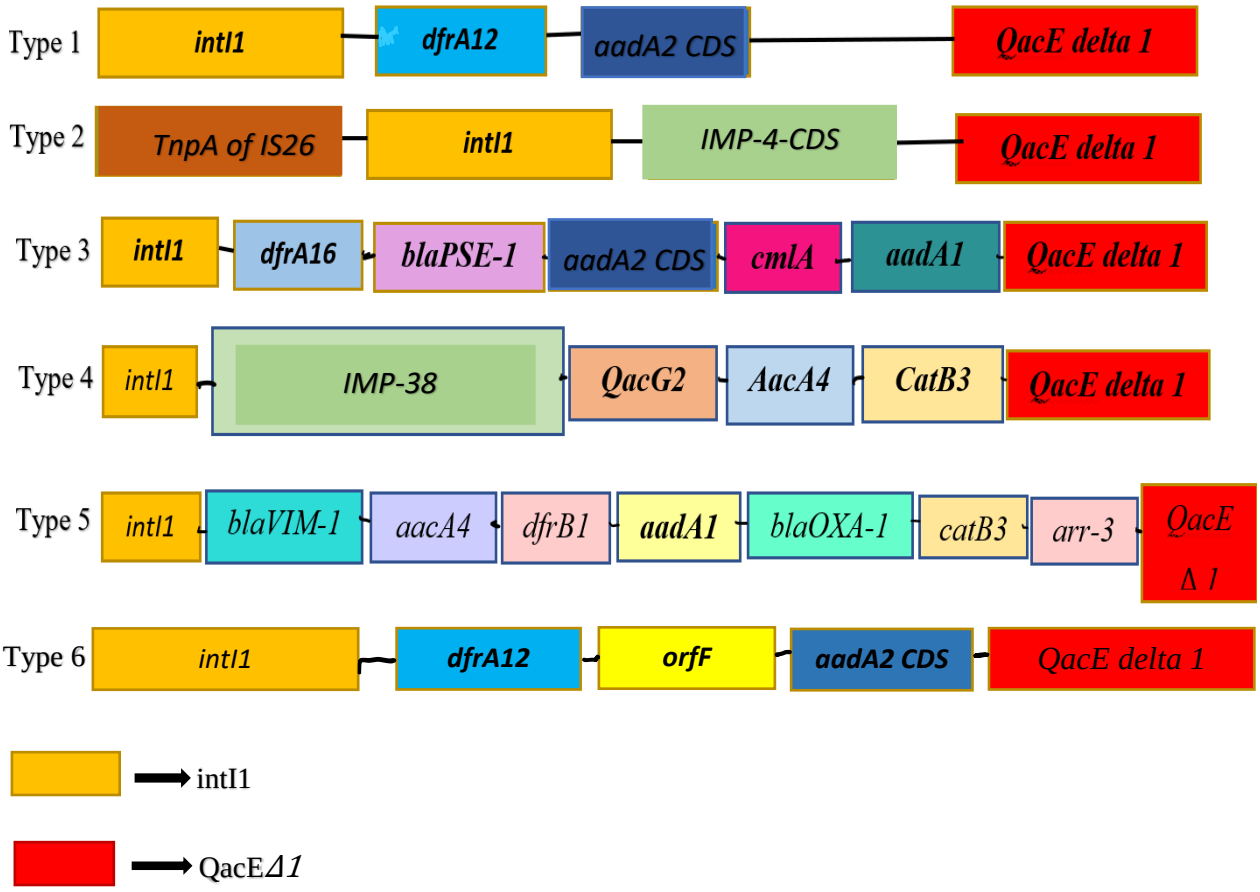


Figure 13: Schematic representation of types of class 1 integrons gene cassette orientation identified between *IntI1* to *QacEdelta1* by Megablast**.**

The consensus sequence of *IntI1* and *QacEdelta1* from Pacbio sequencing, the antibiotic resistance genes such as *blaOXA-1* CDS gene encoding the broad-spectrum beta-lactamase OXA-1 along with *aadA2* cds (i.e. *Pseudomonas* aeruginosa plasmid pKM0509) were identified.

Several uncultured bacteria were also reported from the waste water treatment plant sample. Unclutured bacteria cannot be grown in the synthetic nutrient medium provided by the lab. They require natural complex environment to grow. Presence of *OxA-10*(beta-lactamase giving ceftazidime) between recombinase attI and 59- base element has been observed in the uncultured bacteria from the waste water treatment plant sample as shown in the figure-20

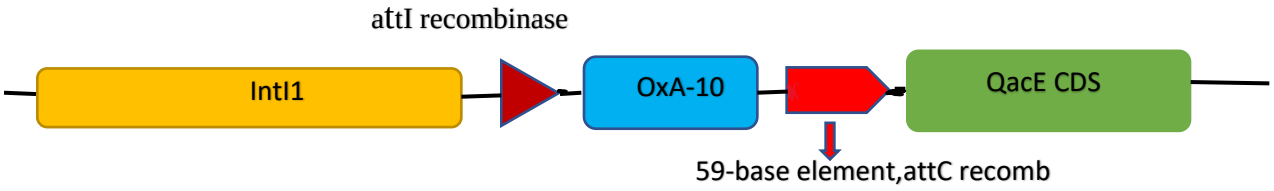


Figure 14: class 1 integrons from Uncultured bacteria strains. *OxA-10*(beta-lactamase giving ceftazidime) between recombinase and 59- base element( Genbank: FJ820140).

## 6. Discussion

The antibiotics resistance genes such as *CatB8, -aadA2, blaOxA-10, IMP-38* were amplified from *IntI1* to *QacEdelta1*, thus the designed primers and the optimization of Long-Range were successful. The combination of inverse PCR and Pac-Bio sequencing was sucessful to amplify the antibiotic resistance genes from Class 1 integrons. However, the combination of long-range PCR with Pac-Bio sequencing was a novel approach to study the ARGs present in the class 1 integrons.designed primers and optimized long range PCR simplified DNA from the wastewater treatment plant has antibiotic resistance genes in class 1 integrons such as *cmlA1* ehich encodes chloramphenicol efflux pump and *IMP-38*-putativereverse transcriptase protein are found between *IntI1* to *QacEdelta1*. However these two genes were reported in class1 integrons from the soil samples but its novel from the Pac-bio sequence analysis of wastewater treatment plant.The class 1 integrons from wastewater treatment plant sample had *dfrA12,* a gene encoding *for* trimethoprim-resistant dihydrofolate reductase. It was 4000kb in length. One of the class 1 integron had type B-3 chloramphenicol O-acetyltransferase, *CatB8* having a score 3365, 99% with identity match *Acinetobacter baumannii* strain 15A34 chromosome. It has been previously reported in municipal wastewater[(75)]

Wastewater treatment is not effective to remove the class 1 integrons because it is not designed to remove bacterial DNA having the antibiotic resistance genes[65]. Moreover, the amount of *IntI1* gene increases with the wastewater treatment process[63,64]. Due to selective pressure, the bacteria acquire class 1 integrons antibiotic resistance genes by horizontal gene transfer and their abundance increases with stages of wastewater treatment[66]

Many antibiotic resistance genes in class 1 integrons such as *dfrA12, -aadA2* from the class 1 integrons of wastewater treatment plant samples are previously reported in *Klebsiella pneumoniae* isolates from a Hospital in China[(43)]. The presence of *the aadA* gene in class 1 integrons is present in the soil samples from poultry in Korea[(44)]

The Long-Range PCR successfully amplified the larger fragments which were more than 4000kb long and in combination with Pac-bio sequencing made it easy and less time consuming to study the gene cassette between *IntI1* to *QacEdelta1*. The combination of Long-Range PCR with Pac-Bio sequencing can be helpful to save time as compared to the previous procedures to sequence the ARGs [47,48,50].

The combination of Long-Range PCR and Pac-bio sequencing is time efficient. The antibiotics resistance genes are successfully amplified from the samples of the wastewater treatment plant using the Long-Range PCR and Pac-bio sequencer. This combination of technique can be used for further analysis of the antibiotic resistance genes present in class 1 Integrons of other samples from areas such as fish farm sediments, soil from hospital area, soil from farming land.

## 7. Conclusion

The wastewater sample is an important reservoir of antibiotic resistance genes. The wastewater treatment plant is not able to remove the antibiotic resistance genes completely even after the multiple processing steps by the wastewater treatment plants. The class I integrons found from the waste water treatment plant carry a high number of antibiotic resistance genes present between *IntI1* to *QacEdelta1*. In the class 1 integron, the antibiotic resistance genes such as *CatB8* resistant to chloramphenicol, *blaOXA-1* resistant to beta-lactamase are acquired through site-specific recombinase. The new antibiotic resistance genes in class 1 integrons such as *cmlA1* which act as a chloramphenicol efflux pump and *IMP-38* a gene encoding for putative reverse transcriptase protein are found from the wastewater samples. The Long-Range PCR saves time and gives DNA amplified products longer than 1500kb – 5000kb. The purified samples from long range PCR can be studied by direct sequencing using the Pac-Bio sequencer. Thus, the future implementations of the above combination of two techniques can be very useful to study the antibiotic resistance genes in the soil and polluted water.

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